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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

OIPE JC63 NOV 10 1999 PATENT & TRADEMARK OFFICE

In re Application of:	)
ROGELJ *et al.*	)
Serial Number: To be assigned	)Art Unit: To be assigned
Filed: Concurrently herewith	)Examiner: To be assigned
For: INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE	)

RECEIVED
28 JAN 2000
Legal Staff
International Division

Assistant Commissioner for Patents
Washington, D.C. 20231

NATIONAL STAGE FILING OF A PCT APPLICATION IN COMPLIANCE WITH PCT ARTICLE 33(1)-(4)-IPEA-US

Sir:

The below-identified communication(s) is (are) submitted in the above-captioned application or proceeding:

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- ☒ Application (33 pgs spec, 19 claims)
- ☒ Declaration
- ☒ Fee Transmittal Form
- ☒ Formal Drawings (6 sheets)
- ☒ Small Entity Statement
- ☒ Preliminary Amendment
- ☒ Check for $48.00
- ☒ Associate Power of Attorney
- ☒ Information Dis. Statement
- ☒ Form PTO 1449
- ☒ Notice of Priority
- ☒ EDC Form

☒ The Commissioner is hereby authorized to charge payment of any fees associated with this communication, including fees under 37 C.F.R. §§ 1.16 and 1.17 or credit any overpayment to **Deposit Account Number 10-0233-UNME-0054-1.**

Respectfully submitted,

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November 9, 1999

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FEE TRANSMITTAL FORM

CLAIMS	FOR	NUMBER FILED	NUMBER EXTRA	RATE	CALCULATIONS
	TOTAL CLAIMS	19-20=	0	x$18 =	$0.00
	INDEPENDENT CLAIMS	2-3=	0	x$78 =	$0.00
	MULTIPLE DEPENDENT CLAIM (S) (if applicable)			+$260 =	$0 00
			BASIC FEE 1.492(A)(4)		$96.00
			TOTAL OF ABOVE		$96.00
	REDUCTION BY 50% FOR FILING BY SMALL ENTITY (note 37 C F R §§ 1 9, 1 27, 1 28)				-$48.00
				TOTAL=	$48.00

1. ☒ A verified statement to establish small entity status under 37 CFR 1.9 and 1.27
   - ☒ is enclosed
   - ☐ was filed in prior application number xx/xxx,xxx and such status is still proper and desired (37 CFR 1.28(a)).

2. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required under 37 CFR 1.16 and 1.17, or credit any overpayment to Deposit Account No. 10-0233-UNME-54.

3. ☒ A check in the amount of $48.00 for the filing fee is enclosed.
   ☐ A check in the amount of $_______ for the assignment is enclosed.

4. ☐ Cancel in this application original claims _______ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

Address all future communications: (May only be completed by applicant or attorney/agent of record)

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☒ Attorney or Agent of Record
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☐ Assignee of Complete Interest
☐ Filed under § 1.34(a)

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	)

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-captioned application, filed concurrently herewith, without prejudice or disclaimer, as follows:

IN THE CLAIMS:

Please amend the claims, without prejudice or disclaimer, as indicated below:

11. (Amended) An inhibitor according to Claim 2 [or 9], wherein one of R or R' is an uncharged H or C_1-C_6-alkyl ligand.

12. (Amended) A method for inhibiting PDI by exposing cells expressing PDI to a compound according to [any one of] Claim[s 1-8] 1 in an amount sufficient to inhibit PDI activity.

14. (Amended) A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to [any one of]

Claim[s 1-8] 1 in an amount sufficient to inhibit viral propagation.

19. (Amended) A method for determining optimum blood concentrations of a PDI inhibitor for treatment of a mammal for a viral infection according to Claim 14 [or 15], comprising admixing a blood sample with PDI inhibitor and assaying for leucocyte L-selectin shedding.

Please add the following claims:

--20. An inhibitor according to Claim 9, wherein one of R or R' is an uncharged H or C_1-C_6-alkyl ligand.

21. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 2 in an amount sufficient to inhibit PDI activity.

22. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 3 in an amount sufficient to inhibit PDI activity.

23. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 4 in an amount sufficient to inhibit PDI activity.

24. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 5 in an amount sufficient to inhibit PDI activity.

25. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 6 in an amount sufficient to inhibit PDI activity.

26. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 7 in an amount sufficient to inhibit PDI activity.

27. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to Claim 8 in an amount sufficient to inhibit PDI activity.

28. A method for treating a mammal for a viral infection propagated by

PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 2 in an amount sufficient to inhibit viral propagation.

29. A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 3 in an amount sufficient to inhibit viral propagation.

30. A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 4 in an amount sufficient to inhibit viral propagation.

31. A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 5 in an amount sufficient to inhibit viral propagation.

32. A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 6 in an amount sufficient to inhibit viral propagation.

33. A method for treating a mammal for a viral infection propagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 7 in an amount sufficient to inhibit viral propagation.

34. A method for treating a mammal for a viral infection propagated by

PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to Claim 8 in an amount sufficient to inhibit viral propagation.

35. A method for determining optimum blood concentrations of a PDI inhibitor for treatment of a mammal for a viral infection according to Claim 15, comprising admixing a blood sample with PDI inhibitor and assaying for leucocyte L-selectin shedding.--

Serial Number:

REMARKS:

Favorable consideration of this application as presently amended is respectfully requested.

The amendments to the claims are requested to remove the multiple dependency. The amendments to claims 11, 12, 14 and 19 are written to put the claims in non-multiple dependent format. Claims 20 through 35 are added to cover the dependency of original claims 11, 12, 14 and 19. New claims 20 through 35 are written in non-multiple dependent format. No new matter has been added by these amendments or by the new claims.

It is respectfully submitted that this application is now in condition for allowance, and favorable action is respectfully solicited.

Respectfully submitted,
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November 9, 1999

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Application for

United States Letters Patent

of

Snezna ROGELJ
Larry A. SKLAR
Robert B. PALMER

for

INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE

INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE

BACKGROUND OF THE INVENTION

The invention relates to anti-thiol reagents which inhibit enzyme activity of cell-associated protein disulfide isomerase (PDI) by oxidizing or blocking PDI active site vicinal thiol groups which normally participate in disulfide bond rearrangement of PDI substrates. Inhibition of this PDI function is particularly useful in blocking PDI-mediated entry of HIV or other virions into a host cell.

The invention further relates to an assay for the identification of such PDI inhibitors based on the discovery that inhibitors of the invention also induce shedding of the leukocyte L-selectin adhesion molecule.

1. Field of Art:

PDI (protein disulfide isomerase) is a constitutive intracellular protein that is also found to be expressed on the surface of many mammalian cell types, including immune system cells, hepatocytes, and platelets. Like other members of the thyredoxin superfamily of proteins, PDI is a multifunctional redox-sensitive protein that catalyzes oxidation-reduction reactions via a vicinal dithiol-dependent disulfide-sulfhydryl interchange between its internal vicinal dithiol (Cys-Gly-His-Cys) active sites and the disulfide bonds of its substrates to promote their reconfiguration. PDI recognizes the side chains of cysteine residues in its substrates, and it is its two vicinal dithiol groups, one or two on each of two identical PDI subunits, that are critical for its enzymatic isomerase function, in particular its broad specificity for correcting the configuration of a large spectrum of proteins as needed. For example, PDI is present in the

endoplasmic reticulum of most cells, where it is believed to mediate co- and post- translational modifications of nascent proteins with incorrect sulfide bonds; it is also present in certain protein complexes such as triglyceride transfer protein complex (MTP) wherein it maintains the complex in a catalytically-active state and inhibits complex aggregation. Membrane PDI catalyzes the cleavage of disulfide bonds during the earliest stages of endocytosis, and activates diphtheria toxin by catalyzing cleavage of this disulfide-linked dimer. PDI also catalyzes the isomerization of thrombospondin (TSP) disulfide bonds, thereby profoundly modulating TSP-ligand binding activity. Both TSP and PDI are released by activated platelets; PDI is also released by degranulated neutrophils (J. Cell Physiol. 144: 280, 1990).

Other known PDI functions include the recognized ability of PDI to modulate certain adhesive interactions. While PDI isomerase activity affects, for example, the adhesive properties of TSP, PDI is additionally a "chaperone" for some proteins by means independent of its catalytic activity. One of these chaperone functions has been attributed to PDI binding in a complex formation with proteins which have a tendency to aggregate in the denatured state. Association with PDI prevents this aggregation by promoting appropriate folding of the associated protein. PDI in MTP complexes inhibits MTP aggregation, and a PDI homolog (cognin) plays a role in the adhesion-dependent aggregation of retinal cells.

2. Discussion of Related Art

Of particular relevance to the present invention is the involvement of PDI in the shedding of the human thyrotropin (TSH) receptor ectodomain (Biochem, 35:14800, 1996). In a two-step process, a matrix metalloproteinase first cleaves the receptor into two subunits (an α-extracellular subunit and a β-transmembrane subunit) linked by a disulfide bridge. The

α-extracellular subunit is then shed from the cell membrane as a result of PDI-mediated reduction of the disulfide bridge(s) connecting it to the β-transmembrane subunit. However, in contrast to the PDI-mediated L-selectin shedding mechanism according to the present invention, the TSH shedding mechanism requires PDI isomerase activity, and inhibition of PDI activity with known PDI inhibitors such as DTNB (5,5'-dithiobis (2-nitrobenzoic acid), bacitracin, or anti-PDI antibodies to prevent the shedding (release) of the TSH α-subunit.

Also of relevance is the known ability of PDI to mediate transmembrane carriage of proteins and virions into cells by rearrangement of their disulfide bonds. For example, the attachment of HIV to its host cell surface receptor CD4 via the viral glycoprotein gp 120 triggers a conformational change in gp 120/gp 41 resulting from a rearrangement of its critical disulfide bonds as catalyzed by PDI. Known PDI inhibitors (e.g., bacitracin, anti-PDI antibodies) block HIV entry into the cell cytoplasm to some extent, but they are very weak inhibitors of PDI isomerase activity in this clinical application (PNAS USA 91: 4559, 1994). The use of another known PDI inhibitor, DTNB (*supra*) to inhibit viral penetration into cells has been described (U.S. Patent 5,532,154 to Brown); however, the recited activity of this compound in preventing HIV entry into cells is attributed by the patentee to inactivation of "virus-derived thiol reductase/protein disulfide isomerase", presumably encoded by and present on the virus itself.

The interaction of arsine oxide with certain proteins having active vicinal dithiol sites which undergo catalytic conversion to disulfides to form stable dithioarsenic derivatives is described in Anal. BioChem 212: 325-334 (1993). This reactivity was used by the authors to separate dithiols from monothiols and also from dithiol-containing proteins with low-affinity for arsine oxide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Regulation of L-selectin shedding. Reduced cell surface PDI constitutively maintains L-selectin in the reduced, non-cleavable conformation. Chemical blockade or direct oxidation of the PDI vicinal dithiol active sites leads to a formation of a critical disulfide bridge within L-selectin molecule. The resulting conformation of L-selectin permits cleavage by the sheddase. In the presence of TAPI, L-selectin shedding is blocked.

FIG. 2. Induction of L-selectin shedding with PAODMPS*.

FIG. 3. Effect of PAO, PAO* and BESA on L-selectin shedding from human neutrophils.

FIG. 4. Effect of PAO on neutrophil adhesion molecules (a) PAO induces L-selectin shedding from neutrophils in a dose dependent manner. Control-4°C represents a neutrophil population that has remained at 4°C since isolation. L-selectin levels were normalized, with the expression on the Control-4°C population representing 100%. Control - 10 min. @ 37°C is an untreated population that underwent a mock 10 minute incubation along with the PAO and/or fMLP treated cells. (b) A phenotypic analysis of PAO treated neutrophils. 100nM PAO does not appreciably upregulate Mac-1 or induce the shedding of other cell surface molecules known to undergo proteolytic cleavage. The mean channel number of fluorescence is reported with the

S.E.M. Experiments were performed twice with duplicate or triplicate samples done in each. (c) *Activity in whole blood.* Peripheral venous blood was diluted 1:10 with HHB buffer and treated with 1 μM PAO for 20 minutes at 37°C. L-selectin expression was measured by the subsequent FACScan analysis of the LDS-751 and Leu8-FITC labeled cells.

SUMMARY OF THE DISCLOSURE

The invention provides cell-surface protein disulfide isomerase (csPDI) inhibitors which block PDI-mediated disulfide rearrangement in PDI substrates. In particular, the invention provides (di)thiol-reactive reagents which react with active site vicinal dithiols of csPDI to inhibit the substrate disulfide bond rearrangement prerequisite for entry of the substrate into the cell.

The invention is in part predicated on the discovery that inhibitors according to the invention also promote shedding of the leucocyte adhesion molecule L-selectin, and screening protocols for identification of inhibitors within the scope of the invention based on this phenomenon are accordingly further provided.

The inhibitors of the invention are particularly useful for denying viruses requiring disulfide bond rearrangement for transmembrane passage to access host cell DNA for replication. The invention accordingly provides methods for inhibiting replication of such

viruses, notably gag retroviruses including HIV virotypes, by disrupting their PDI-mediated cell entry mechanisms.

The invention additionally provides a two-pronged approach for prevention and treatment of PDI-mediated viral infection based on the observed contemporaneous inducement of L-selectin shedding and the inhibition of viral cell entry by the inhibitors of the invention.

Pharmaceutical compositions containing the inhibitors of the invention andmethods for treating or preventing viral infection in humans or other mammals, including periodic elevations of HIV or other viremia, are also provided. Combinations of these compositions and methods with other viral therapies are useful.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, cell-surface PDI (csPDI) isomerase activity is effectively inhibited by thiol blocking agents (inhibitors) which covalently or non-covalently cross-link two or more free vicinal sulfhydryl groups of one or more PDI active site peptide sequences to form complexes stable in the cell environment. The -SH groups of the cysteine residues in the sequence Cys-Gly-His-Cys are exemplary. The inhibitors are preferably highly selective for PDI vicinal sulfhydryls and have sufficient affinity for these groups to compete successfully with the ligand to be denied access to these sites and prevent PDI-mediated isomerization of its disulfide bonds and its consequent reconfiguration for undesired biological activity. The sequence of PDI is known (Nature 317:6034; 267, 1985). Herein, "csPDI" and "PDI" are used interchangeably unless otherwise noted.

Inhibitors according to the invention are useful for inhibiting thiol-mediated csPDI isomerase activity with proper selection of inhibitors, and possibly one or more non-isomerase activities, such as the afore-mentioned "chaperone" activity. This includes both presently-known PDI isomerase and other activities and other cell-associated PDI activities yet to be discovered.

In one embodiment, the PDI inhibitors of the invention inhibit virion cell entry mediated by csPDI expressed on the plasma membrane of potential host cells, particularly gag retroviruses such as HIV virotypes. As described in further detail below, PDI inhibitors useful for this application can be directly identified by appropriate NIH protocols such as those described for HIV *infra*. Alternately, according to the invention, potentially useful PDI inhibitors are identified by screening for their ability to inhibit or induce L-selectin shedding. The ability of the L-selectin shedding assay of the invention (Examples) to identify inhibitors of thiol-mediated csPDI activity is demonstrated in detail *infra*. This assay is based on the observation that a blockade of leucocyte csPDI oxido-reductive function induces a release of L-selectin, a cell surface adhesion molecule, and that the rate of L-selectin release is a direct reflection of the efficacy of the csPDI inhibitor. Therefore, measuring the amount of L-selectin released from leucocytes within a certain time period provides information regarding the potency of the putative csPDI inhibitor.

A detailed description of L-selectin shedding phenomena is given in J. Immun. 156:3093-3097, 1996, the entire contents and disclosure of which are herein incorporated by reference. Briefly, the L-selectin adhesion molecule mediates leucocyte recruitment to inflammatory sites and lymphocyte trafficking through the peripheral lymph nodes. In response to leucocyte activation, L-selectin is proteolytically released (shed) from the cell surface,

disabling leucocytes from the subsequent L-selectin-dependent interactions. L-selectin shedding is sensitive to sulfhydryl chemistry and PDI regulates the susceptibility of leucocyte L-selectin to shedding promoted by (di)thiol oxidizing or blocking reagents according to the invention. In contrast to known prior art shedding mechanisms such as those for TSH (*supra*), csPDI constitutively acts on L-selectin to maintain its disulfide bonds in a reduced, non-cleavable state; blockade of PDI permits reversion of these bonds to the oxidized, cleavable conformation for shedding. Thus, shedding is promoted by the instant inhibitors in direct relationship to the effect of the inhibitors on blocking csPDI function (FIG. 1). Since L-selectin is also present on lymphocytes and mediates their entry into peripheral lymph nodes, L-selectin shedding according to the invention also inhibits the movement of virally-infected lymphocytes into these nodes, preventing the establishment of viral reservoirs responsible for spreading the infection to uninfected lymphocytes. In chronically infected individuals, the inhibition of nascent, uninfected lymphocyte trafficking through the lymph node viral reservoirs would further prevent the exposure of these cells to the virus, and therefore the internal spread of HIV. Finally, by inducing the shedding of lymphocyte L-selectin, these reagents would ameliorate the lymphoadenopathy thought to be due to an increase in L-selectin expression, and therefore homing to the lymph nodes, by the abortively HIV-infected lymphocytes.

PAO (phenylarsine oxide), a trivalent arsenical inhibitor of the invention interacts with PDI vicinal dithiols and is most potent in inducing rapid shedding of L-selectin from isolated neutrophils, eosinophils, and lymphocytes, as well as from neutrophils in whole blood. PAO does not cause cell activation, nor does it interfere with integrin function or alter the expression of several other cell surface molecules at the lower concentrations that effectively induce L-selectin shedding. Further, PAO is not required to enter the cell to induce L-selectin shedding. TAPI, which has previously been shown to inhibit activation-dependent L-selectin

shedding, is also capable of inhibiting PAO-induced L-selectin shedding. The Snezna L-Selectin Assay of the invention has been validated with human leucocytes but is anticipated to work as well with leucocytes of other species. In all cases, species-specific anti-L-selectin antibodies should be used for labeling. Although the assay can be carried out with either neutrophils or with lymphocytes, neutrophils appear to provide a faster and a more sensitive L-selectin shedding response. PDI inhibitors identified by this protocol are useful in the inhibition of PDI according to the present invention.

Inhibitors of the invention comprise agents which form stable complexes or derivatives by covalent or non-covalent binding with one or more active thiol groups of PDI to inhibit catalytic rearrangement of substrate disulfide groups. Both monothiol inhibitors (which inactivate single thiol groups) and dithiol inhibitors (which cross-link two thiol groups) are useful, especially inhibitors specific to PDI vicinal sulfhydryl groups, and act by blocking or oxidizing the groups. Preferred inhibitors for *in vivo* use comprise membrane impermeable inhibitors [denoted herein with an asterisk (*)], as this avoids toxicity resulting from undesirable effects of cell-permeable inhibitors on intra-cellular processes. More preferred for *in vivo* uses are asterisked inhibitors efficacious in evaluation protocols at relatively low concentrations (i.e., concentrations with respect to each other).

Typically, dithiol inhibitors will have greater efficacy as inhibitors, as they have been found to have greater potency at lower concentrations. This is important, as many of the inhibitors are potentially toxic to cells at high concentrations, especially cell-permeable inhibitors. Vicinal dithiol-reactive inhibitors will have a preference for the vicinal (closely spaced) dithiol (reduced cysteine) sites such as those found in the active sites of PDI. It is known that the dithiol-reactive reagents are much less likely to interact with monothiol sites.

(Reactivity with monothiol sites should generally be avoided as it would lead to loss of specificity and an increase in cytotoxicity.) Dithiol reactivity thus enhances specificity and potency while minimizing cytotoxicity. Using dithiol reagents such as PAO derivatives offers an additional advantage: they can be selectively removed from a target protein such as PDI with DMP (British Anti-Lewisite, a clinically-known heavy metal antidote) or the related (membrane-impermeable) DMPS, once the desired effect has been achieved. Such a reversal can be carried out if PAO(*) toxicity becomes an issue and PAO(*) needs to be flushed-out from the patient.

Exemplary inhibitors useful in the practice of the invention are set forth in TABLE I, below.

TABLE I

COMPOUNDS THAT INDUCE L-SELECTIN SHEDDING FROM NEUTROPHILS

100% LOSS from cell surface in 10 min at 37 deg. at
the approximate concentration:
(membrane-impermeable inhibitors are marked with *)

Thiol Blocking and Oxidizing reagents

PAO	-1 uM -	vicinal dithiol specific
PAO-DNP	-1 uM -	DNP added far from AsO group
Aminophenylmercuric acetate ..	10 uM -	monothiol reactives activates MMPs
Nitroblue tetrazolium.................	100 uM-	superoxide scavenger and oxidant
Hydrogen peroxide	150 uM -	oxidant, effect inhibitable by excess DMPS
Monobromobimane mBBr.........	100 uM -	thiol blocker (fluorescent as bound)
Dibromobimane bBBr	100 um -	dithiol reactive, spacing differs from PAO
*DIDS	200 uM -	thiol and amino-group reactive
N-ethylmaleimide	250 uM -	thiol blocker
*Quaternary bromobimane qBBr	300 uM -	membrane-impermeable mBBr
Iodoacetate IA	300 uM -	thiol blocker, also depletes energy
*Mersalyl acid	500 uM -	thiol-reactive via - HgOH
Thimerosal	500 um -	thiol blocker, used as preservative

*DTNB	1 mM -	thiol-blocker
PMSF	1 mM -	attacks and blocks - SH (and OH) groups
Diamide	1 mM -	thiol oxidizer/crosslinker, ADA analog
Azodicarbonamide	1 mM -	blocks HIV infectivity, flour additive
Iodoacetamide	10 mM -	thiol reagent, low efficacy (vs. IA) surprising
Iodosobenzoate	1 mM -	A dithiol oxidizing reagent just like PAO, but with Iodine instead of arsenic, causes direct oxidation of dithiols

Dithiol Reactive Ions:

Arsenic (As203)	50 uM
Cadmium (CdC12)	1 mM
Antimony (Sb203)	50 uM

Other Reagents and Conditions:

*Dehydroascorbate	I M -	specifically and only reduced by PDI or glutaredoxin, would lead to net oxidation of PDI
Chlorpromazine	50 mM -	blocks HIV infectivity at this concentration
Methoxychlor	50 uM -	a pesticide, a calmodulin antagonist
DDT	50 uM -	a pesticide, a calmodulin antagonist
Disulfiram (Antabuse)	50 uM -	drug that induces alcohol aversion
*Thioredoxin	50 ug/ml -	reductant at cell surface, poor isomerase, has biphasic effect on shedding
*Protein Disulfide Isomerase	20 ug/ml -	effective isomerase at cell surface
Somatostatin	200 uM -	peptide PDI inhibitor
UV irradiation	10 min -	produces ROS, effect TAPI inhibitable

As alluded to *supra*, the inhibitors of the invention are especially useful *in vivo* for blocking entry of virions into targeted host cells by blocking an essential PDI-mediated step of their transmembrane process. Since some of the inhibitors useful in this process such as PAO also inhibit or induce L-selectin shedding from lymphocytes and thus inhibit lymphocyte entry into peripheral lymph nodes, the establishment of reservoirs of transfected lymphoctyes is concurrently inhibited. This is an important advantage when treating viral infections characterized by lymphocyte infection such as HIV, since HIV-infected cells can each produce

billions of virions daily and it is the subsequent entry of these virions into uninfected cells which propagates the disease.

Most of the inhibitors identified by the inventors to date, including cadmium, and trivalent arsenical and antimonial compounds work by blocking the vicinal cysteines in PDI active sites; however, some inhibitors may work by blocking PDI activity by a mechanism that is different from the thiol-mediated blockade of the Cys-Gly-His-Cys active sites. The inhibitors are generally not cell-specific (unlike, for example, fMLP for which CHO and lymphocytes are receptor negative), and are selected as the application requires as described herein. Cell-membrane impermeable inhibitors are typically selected for applications requiring minimization of toxicity as are the dithiol and dithiol-specific inhibitors, as these tend to be efficacious at lower relative concentrations. Monothiol and/or cell-membrane permeable inhibitors are, however, useful in the practice of the invention and may prove equal or superior to dithiol inhibitors in applications where a slight increase in cell toxicity is not a critical factor.

Pharmaceutical compositions containing the inhibitors of the invention are useful as prophylactics for immediate treatment on exposure to HIV or other PDI-dependent viruses and for treating established viral infection, including periodic elevations of HIV or other viremia. Treatment according to the invention with other therapies is contemplated.

Compositions for parenteral administration comprise aqueous solutions of the inhibitors of the invention in an amount sufficient to provide a blood concentration of about 1 μM or less in whole blood. More potent inhibitors are contemplated to be effective at concentrations of about 0.1gM or less. Suitable concentrations are readily determined by combining an inhibitor with a blood sample and selecting a concentration that induces PDI shedding from leucocytes,

usually within about 20 minutes to 2 hours, depending upon inhibitor concentration. For intravenous administration, compositions comprising inhibitor and (at least mostly) water as solvent in concentrations of about 35 mg/ml of solvent are generally effective.

For example, 1 uM PAO (i.e., about 0.17 mg PAO/liter of blood) blocks PDI in whole human blood to the extent that 50% L-selectin shedding from neutrophils is induced in about 20 min. 10 uM (i.e., about 1.7 mg PAO/liter of blood) should induce complete shedding within about 20 min. Thus, administering sufficient PAO to attain 10 uM concentration of PAO in blood should fully block leucocyte csPDI. Since only a fraction of total body weight is blood (assume 10 liters in a 50 kg human), then 17mg of PAO is required for this effect. This translates to 17 mg/50 kg or about 0.35 mg PAO/kg body weight. A 100 kg human thus would require about 35 mg of PAO to attain "instantaneous" 10 uM blood concentration. PAO at these low concentrations is soluble in most aqueous media (the initial high concentration stock in DMSO can be diluted into aqueous buffer for *in vivo* use).

A recommended procedure for *in-vivo* administration of PAO comprises preparing an injectable (for example, i.v.) aqueous stock of PAO containing 35 mg PAO/ml PBS. Depending on the weight of the patient, an appropriate volume is injected (e.g., a 70 kg person would receive about 0.7 ml of the preparation). To avoid local toxicity, this stock preparation can be diluted further and proportionally larger volume injected slowly to attain the same blood concentrations in a comparable period of time.

Similar calculations are carried out for the various PAO* inhibitors, and based on the predetermined concentration required to induce full shedding of L-selectin in whole blood. For example, 10 uM PAO* is required to obtain about the same results as 1 uM PAO, in whole

blood. The required concentrations for treatment are thus 10 times higher, i.e., 3.5 mg PAO*/kg body weight. The injectable stock preparation should then be made up as 350 mg PAO*/ml PBS; a 70 kg human would need 0.7 ml of this stock preparation. In practice, lower concentrations might suffice, as these are high-end estimates. Concentrations should be kept to a minimum sufficient for effectiveness to minimize toxicity.

Of great advantage is that the inhibitors are readily soluble in aqueous media at the concentrations needed for effective administration for inhibiting PDI isomerase activity and/or virion entry into cells. Compositions can also be administered orally; oral compositions comprising excipient(s) and inhibitor in amounts which provide blood concentrations as described above are useful in the practice of the invention.

EXAMPLES

I. Detection of L-selectin release:

L-selectin release can be measured either as a loss of L-selectin from the cell surface using a fluorescence activated cell sorter (FACS), or it can be measured as an increase of the released, soluble L-selectin in the cells' supernate (using, for example, an ELISA).

A) Using FACS Analyzer:

Using this method, cell-associated L-selectin is measured. Using fluorescently labeled anti-L-selectin antibody, the cell surface L-selectin is tagged. PDI inhibition causes L-selectin and its tag to be released from the cell surface. This results in a loss of cell-associated fluorescence.

There are two, essentially equivalent, methods for detecting the release of L-selectin from leucocyte cell surfaces.

In the first method, cells are first treated with the inhibitor and then samples are labeled individually for L-selectin that remains on the cell surface. In the second method, the cells are prelabeled with fluorescent anti-L-selectin antibody and then treated with the putative PDI inhibitor. Prelabeling of the cells has only a minor accelerating effect onL-selectin shedding when the treatment tines are in the range of 10 min. The temperature control sample, lacking any drug treatment, reflects this "spontaneous" shedding when compared with a prelabeled sample that was kept on ice. This method, however, permits the preparation of a large volume of uniformly prelabeled substrate cells for the assay, minimizes inter-sample variation, and is less labor intensive. Prelabeling of cells with a fluorescent anti-L-selectin antibody does not interfere with PDI inhibition or L-selectin shedding.

In either case, the cells are labeled with a fluorescent (e.g., FITC or PE) anti-L-selectin antibody on ice for 45 minutes at a concentration in the range of 1mg/ml (see specific example below).

B) Using a soluble L-selectin (sL-selectin) ELISA.

This assay was validated using the Bender MedSystems (Boehringer Ingelheim Group, Vienna, Austria) sL-selectin ELISA kit. However, a much better source of sL-selectin ELISA kits is Endogen (Endogen, Inc. Woburn, MA). Measuring sL-selectin using ELISA rather than the FACScan analysis is a much more convenient method for screening large numbers and

concentrations of various putative PDI inhibitors. Moreover, smaller numbers of leucocytes are required for this method. The one advantage of FACScan analysis over the ELISA is that toxicity to the cells is readily noted an the FSC/SSC FACScan output. Since acute toxicity could result in a PDI-independent loss of L-selectin release, selected sample analysis should be confirmed using the FACScan method.

Final validation of reagent PDI inhibitory activity is carried out *inter alia*, using purified PDI protein (Sigma) and one of the several standard PDI activity assays. One example of such an established PDI assay is based on the ability of PDI to renature, and thus restore activity to, RNAse A with scrambled disulfide bonds (Sigma) (Methods in Enzymology, 251:397-406, 1995). Only properly disulfide-bonded RNAse A is able to degrade RNA. The increase in absorbance at 260nM is a reflection of RNAse activity. A control reaction mix containing only scrambled RNAse without PDI provides for a measure of the uncatalyzed RNAse renaturation. PDI inhibitors abolish RNAse renaturation and are displayed as a loss of 260nM absorbance as compared to control. This method, while highly specific and accurate, is very labor intensive and would prove very expensive for large-scale screening purposes.

II. Preparation of PDI inhibitors:

The putative PDI inhibitors are preferably dissolved in an aqueous solvent. PAO solutions (10mM stock) are prepared in DMSO (Sigma Chemical Co.) and gently heated until PAO goes into solution. Other potential inhibitors of PDI are also dissolved in DMSO. DMSO concentration in the assays should not exceed 1% final volume; above this concentration, DMSO may affect L-selectin shedding. Dioxane should not be used, as it itself induces L-selectin release.

III. Discrimination between PDI inhibition and cell activation:

Activation of neutrophils with a number of biologicals such as fMLP, PAF or LPS, results in the shedding of L-selectin in a manner that does not depend on a direct blockade of PDI activity. Since cell surface increase of Mac-1 integrin, a cell adhesion molecule, (CD18/CD11b) is characteristic of cell activation, reagents which act directly on PDI should not promote the upregulation of Mac-1 on neutrophils. The levels of Mac-1 can readily be measured by flow cytometry using fluorescently labeled anti-Mac-1 antibodies. Useful PDI inhibitors are preferably not cell-type specific. For example, fMLP and LPS only affect neutrophils but not lymphocytes, while preferred PDI inhibitors affect L-selectin shedding from both cell types.

KNOWN CONCEPTS:

1) Direct assay for measuring purified PDI activity. (Freeman RB, Hawkins HC, and McLaughlin SH, 1995 "Protein Disulfide Isomerase", Methods in Enzymology 251:397-406, 1995 incorporated herein by reference).

2) Flow cytometric analysis of cell associated L-selectin. (Bennet TA, Lynam EB, Sklar LA, and Rogelj S. "Hydroxamate-base metalloprotease inhibitor blocks shedding of L-selectin adhesion molecule from leucocytes; functional consequences for neutrophil aggregation", J. Immunol. 156(9):3093-7, 1996, incorporated herein by reference).

3) ELISA for quantitation of soluble L-selectin. (Spertini O, Schleiffenbaum B, White-Owen C, Ruiz P Jr., and Tedder TF, "ELISA for quantitation of L-selectin shed from

leucocytes *in vivo*." J. Immunol. Methods 156(l):115-23, 1992, incorporated herein by reference).

EXAMPLE I

Neutrophil or lymphocyte isolation: Human venous blood was collected from healthy volunteers into sterile syringes containing heparin (10U/ml blood, Elkins-Simms Inc., Cherry Hill, NJ). The blood was separated on Mono-Poly resolving media (ICN Biochemicals, Aurora, OH) by centrifugation of 500g for 22 minutes at 18°C. The granulocyte and mononuclear (for lymphocytes) layers were collected separately and washed in HHB buffer (110mM NaCl, 10mM KC1, l0mM glucose, 1mM $MgCl_2$ and 30 mM HEPES, pH 7.40), then pelleted at 400g for 10 minutes. The cells were resuspended in HHB buffer containing 0.1% human serum albumin (HSA; Armour Pharmaceutical Co., Kankakee, IL) and 1.5 mM CaC l_2, at 10^6 cells/ml and kept on ice. The buffer was depleted of endotoxin by affinity chromatography over columns containing polymyxin B sepharose (Detoxi-gel, Pierce Scientific, Rockford, IL) and autoclaved for one hour. All plastic-ware was autoclaved for at least 45 minutes.

Snezna L-Selectin shedding Assay: Cells suspended in HSA and $CaCl_2$ containing HHB at 10^6 cells/ml were immunofluorescently labeled for 45 min on ice with Leu-8-FITC (IgG2a; Becton-Dickinson Monoclonal Antibodies, Lincoln Park, NJ), a fluorescent mAb which recognizes L-selectin, at a final concentration of 0.625 μg/ml. 200 μl of this prelabeled cell suspension is used for each sample. The assay is preferably carried out in duplicate and contains:

a) Ice control.
b) Temperature control.
c) Solvent control: (b) + solvent of the drug to be tested.

d) fMLP at 100nM.
e) a known PDI inhibitor, e.g. PAO or DTNB.
f) Sample drugs at various concentrations (optional).

Samples (b) onwards were placed into a waterbath at 37°C, for about 10 minutes. The reaction was terminated by placing and keeping the samples on ice. The relative expression of the receptors was quantitated using a FACScan Flow Cytometer (such as Becton-Dickinson).

Controls: To show that cells are viable and normally responsive, neutrophils were activated with fMLP (formyl-methionyl-leucyl-phenylalanine; Sigma chemical Co., St. Louis, MO) for 10 min at 37°C at a final concentration of l00nM. This releases at least 90% of all cell surface L-selectin as measured by the loss of fluorescently labeled L-selectin from neutrophils using FACScan. Lymphocytes do not respond to fMLP. Lymphocytes do shed their L-selectin in response to phorbol ester PMA (100nM), but do so only slowly (~30 min). To show that leucocytes respond normally to PDI inhibitors, the response of both neutrophils and lymphocytes to μM concentrations of phenylarsine oxide (PAO, Sigma) was measured: Neutrophils shed most of their L-selectin in response to 1μM PAO within 10 min; lymphocytes required 5μM PAO to shed their L-selectin within 20 min. Alternatively, a known blocker of PDI, 5',5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Sigma), can be used at 1mM concentration as a positive control.

To check for cell activation, the neutrophils are assayed for the characteristic increase in the cell surface Mac-l expression. This is done by carrying out the above L-selectin shedding assay, but using unlabeled cells. At the end of the 37°C incubation period, neutrophils are placed on ice and Leu-15-PE (IgG2a; Becton-Dickinson Monoclonal Antibodies), a fluorescent mAb which recognizes the α-subunit (CD11b) of Mac-l, is added at the final 1.25 μg/ml

concentration. Samples are incubated with the antibody on ice for 45 minutes, and expression of the receptors thereafter quantitated with the FACScan. An fMLP-treated sample served as a positive control; samples treated with fMLP increased their cell surface expression 3-10 fold compared to the ice control.

The effect of the putative PDI inhibitors on other cell surface molecules was further assessed using either analogous direct immunofluorescence or indirect immunofluorescence. For example, after the treatment of unlabeled cells with the appropriate reagents, the cells were incubated for 40 minutes at 4°C with appropriate antibodies. The antibodies against CD14, CD16 (both at 10 μg/ml; Dako Corporation, Carpinteria, CA), CD43 (8 μg/ml; IgG2a; Camfolio (Becton-Dickinson), San Jose, CA), CD54 (8 μg/ml; Biosource International, Camarillo, CA), PSGL-1 (PL1;IgG1; 10 μg/ml; Dr. Rodger McEver, University of Oklahoma). After incubation the cells were washed by centrifugation for 10 minutes at 400g at 4°C. The second antibody, goat-anti-mouse IgG-FITC (GAM-FITC) polyclonal Ab (Becton-Dickinson Antibodies, Lincoln Park, NJ) at a concentration of 6.25 μg/ml was added and cells were incubated an additional 20 minutes at 4°C. After a final wash, the specific labeling for each antibody was analyzed by flow cytometry.

Time course experiments. For these experiments, isolated neutrophils or lymphocytes were warmed to 37°C and a zero point sample was withdrawn and placed on ice. The putative PDI inhibitor was then added. Cell samples were withdrawn at one minute intervals and placed on ice. The cells were then labeled for 40 minutes with Leu8-FITC on ice and L-selectin expression measured by the FACScan.

EXAMPLE II

General Protocol for Directly Assessing the Ability of PDI Inhibitors to Block HIV Entry into Cells

The protocol used by NIH, Department of Health and Human Service "Anti-HIV Drug Testing system" *infra* is designed to detect agents acting at any stage of the virus reproductive cycle. As is pointed out in this description of the test, certain compounds may not show activity in this test. Inhibitors of PDI, such as PAO and its derivatives, act at the stage of the viral entry which occurs within the first few (2-3) hours of the contact between the viral stock and the target cells. To assay for the specific impact of a drug on viral entry, both the drug and the free virion should be removed from the cells after the infection period. The decrease in the number of the infected cells after the 6 days incubation period will be a reflection on the drug's inhibition of viral entry. This variation on the NIH protocol will additionally eliminate the potential toxic effects associated with the long-term exposure of the drug. The impact of inhibitors on HIV entry may otherwise be obscured by such long-term toxicity. In the case of PAO/PAO* a long-term toxicity was anticipated and in spite of the superimposition of long-term toxicity onto the outcome, PAO* did show anti-HIV activity in this assay. It is expected that about ~10uM PAO* will show a complete blockade of HIV entry when the above steps are followed in the protocol. PAO and other analogues are likely to have a similar effect.

Knowledge that a reagent (a PDI inhibitor) is effective at inhibiting the viral entry is of great value; and changes in drug design according to known principles can subsequently minimize potential toxicities. Since the target of inhibition is PDI, a cell surface protein, this may in many cases only require making the drug less membrane permeable. This is true in the case of PAO, which, when made less membrane permeable (PAO*), nonetheless shows anti-HIV activity. Moreover, there are clinical situations which may require only a short

exposure to the drug which blocks HIV entry and where long-term treatment may not be necessary. One example would be treatment of an individual only recently infected with HIV, as by a stick with an infected needle.

NIH Anti-HIV Drug Testing System

The procedure‡ used in the National Cancer Institute's test for agents active against human immunodeficiency virus (HIV) is designed to detect agents acting at any stage of the virus reproductive cycle. The assay basically involves the killing of T4 lymphocytes by HIV. Small amounts of HIV are added to cells, and two cycles of virus reproduction are necessary to obtain the required cell killing. Agents that interact with virions, cells, or virus gene-products to interfere with viral activities will protect cells from cytolysis. The system is automated in several features to accommodate large numbers of candidate agents and is generally designed to detect anti-HIV activity. However, compounds that degenerate or are rapidly metabolized in the culture conditions may not show activity in this screen. All tests are compared with at least one positive (e.g., AZT-treated) control done at the same time under identical conditions.

The Procedure:

1. Candidate agent is dissolved in dimethyl sulfoxide (unless otherwise instructed) then diluted 1:100 in cell culture medium before preparing serial half-$\log_{10}$ dilutions. T4 lymphocytes (CEM cell line) are added and after a brief interval HIV-1 is added, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound serve as a toxicity control, and infected and uninfected cells without the compound serve as basic controls.

2. Cultures are incubated at 37°C in a 5% carbon dioxide atmosphere for 6 days.

3. The tetrazolium salt, XTT, is added to all wells, and cultures are incubated to allow formazan color development by viable cells.

4. Individual wells are analyzed spectrophotometrically to quantitate formazan production, and in addition are viewed microscopically for detection of viable cells and confirmation of protective activity.

5. Drug-treated virus-infected cells are compared with drug-treated noninfected cells and with other appropriate controls (untreated infected and untreated noninfected cells, drug-containing wells without cells, etc.) on the same plate.

6. Data are reviewed in comparison with other tests done at the same time and a determination about activity is made.

‡Weislow, O.W., Kiser, R., Fine, D., Bader, J., Shoemaker, R.H., Boyd, M.R.: New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. 81;577-586, 1989.

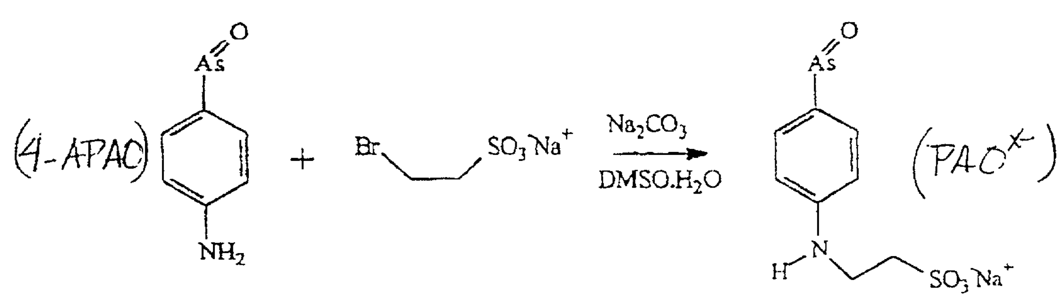
EXAMPLE III

1. Preparation of PAO*

Membrane impermeable derivatives of PAO were designed, prepared, and tested. These derivatives, referred to herein as PAO*s, have a charged ligand attached to the aromatic amine of 4-aminophenylarsenoxide (4-APAO). The charge on this terminal ligand is believed to prevent the molecule from passing through the cell membrane.

A number of potential synthetic PAO* targets exist. In the planning of these preparations, the difficulties of working with organometallic reagents must be noted. First, conditions must be selected so that the arsenic is not inadvertently oxidized from As(III) to As(V). Second, because of the metal content as well as the charged portion of the PAO* molecule, many standard organic chemistry purification and characterization techniques are inoperable.

The first PAO* derivative was prepared through the reaction of 4-APAO with 2-bromoethanesulfonic acid sodium salt in 1:1 DMSO:water. This was carried out in the presence of aqueous Na_2CO_3 at 90° for 8-12 hours.

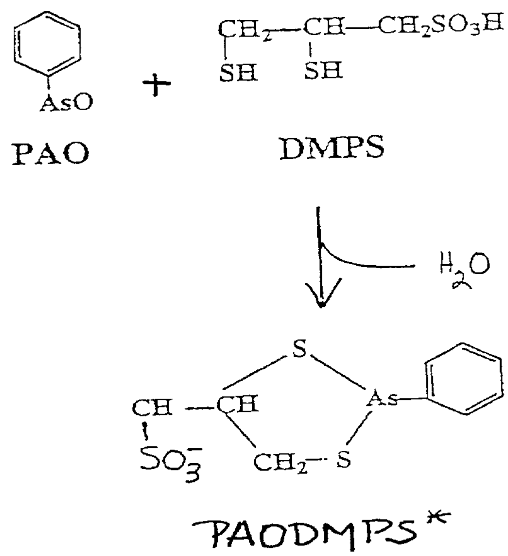


Column and thin layer chromatographies for purificationand purity assessment do not work with this system. Gas chromatography-mass-spectrometry as well as more direct mass spectrometry ionization methods have also proven ineffective due to the low volatility of the

tested compound. Reversed phase HPLC using ocratic water and UV detection provided a reasonable assay for this first PAO* derivative. Along with proton NMR analysis, data confirmed both the structure and purity of PAO*.

2. Additional Versions of PAO*

An additional membrane-impermeable derivative of PAO*, PAODMPS* was prepared according to the following reaction scheme. The compound was shown to induce L-selectin shedding (Fig. 2).



Following the successful purification of this initial PAO* derivative, other PAO* derivatives are prepared and tested.

The following chemical modifications are particularly interesting:

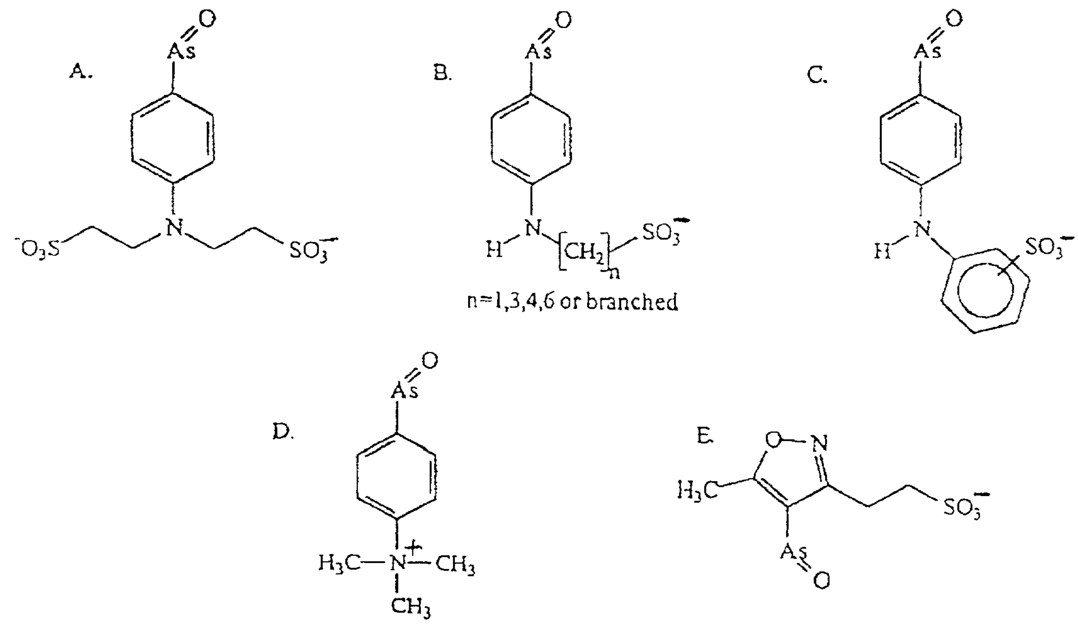
A) The addition of more than one charged entity to the prototypical PAO molecule may impart a further decrease in membrane permeability. Structure A has two anionic SO^3- groups.

B) The addition of alkyl chains of various lengths between the amino nitrogen and the charged group may impart varying degrees of partial membrane impermeability (139). The longer chains may allow the aromatic portion of the compound to permeate the membrane while the charged portion of the molecule remains outside the membrane. Various chain lengths allow for various depths of penetration through the membrane (Structure B).

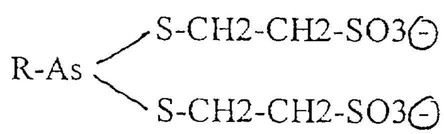
C) Additional control over molecular geometry may be obtained by the incorporation of a more rigid aromatic ligand bearing the charged entity (140). This allows for a more specific control over sterics and electronics than is allowed through the use of simple alkyl chains (Structure C).

D) Negatively charged ligand: A quaternary ammonium salt imparts a positive charge to the ligand. The implications of positive versus negative charges on the PAO* ligand have not yet been examined in this system (Structure D).

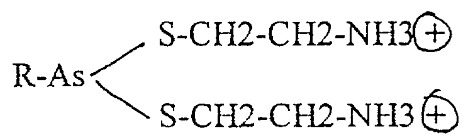
E) The phenyl ring in 4-amino PAO can be replaced with hetero-aromatic substituents, such as an isoxazolyl ring. The isoxazole is well known in medicinal chemistry to be amenable to a variety of substitution patterns (141, 142). Furthermore, an isoxazole can be heterolytically cleaved to the acylaziridine using photochemistry and therefore may be useful as a photoaffinity probe (Structure E) (143). Examples of A-E and ligands F-H are illustrated below:



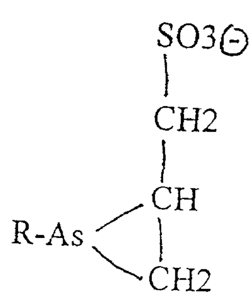
F) Beta-mercaptopropane sulfonic acid:



G) Cysteamine HCl:

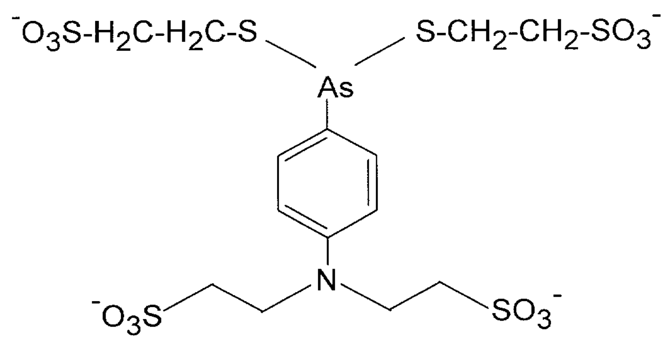


H) Dimercaptopropane sulfonic acid:

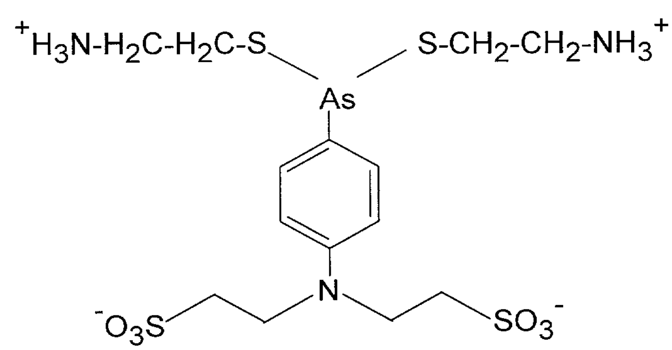


Examples of derivatives of A-E additionally conjugated via the arsenite to the different ligands F-H, are illustrated below:

Derivative of A conjugated with F:

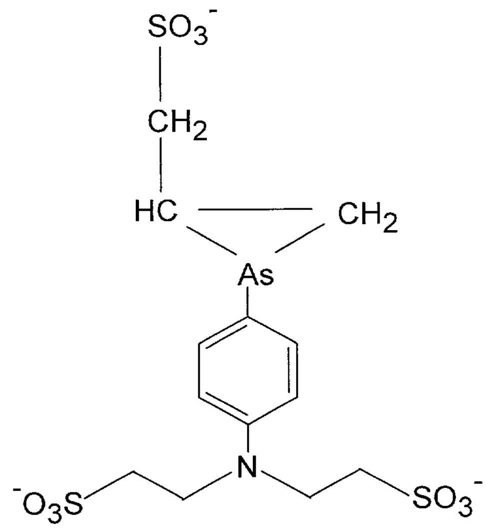
I) 

Derivative of A conjugated with G:

J) 

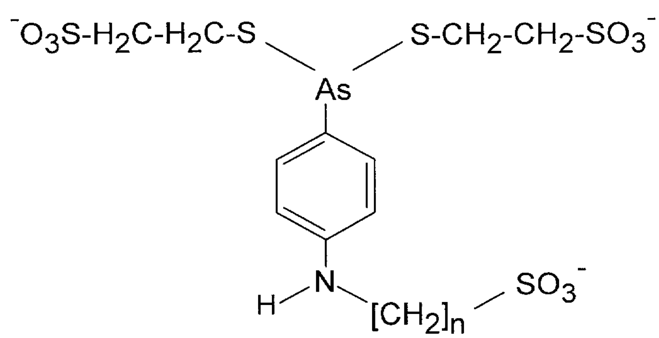
Derivative of A conjugated with H:

K)



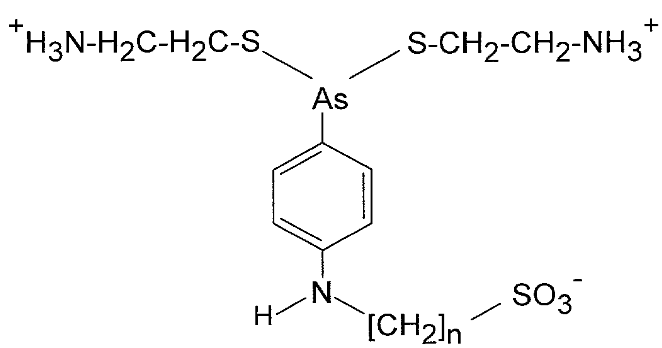
Derivative of B conjugated with F:

L)

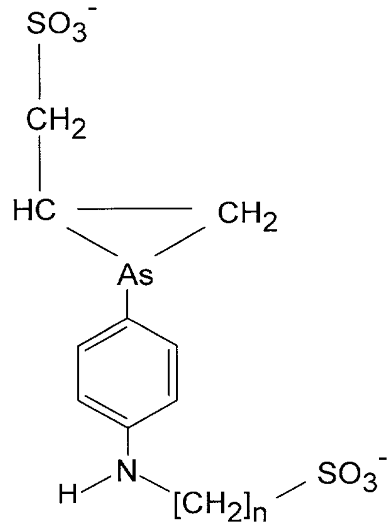


Derivative of B conjugated with G:

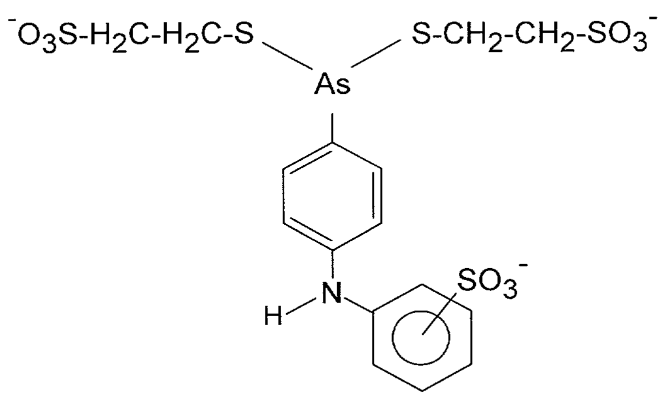
M)



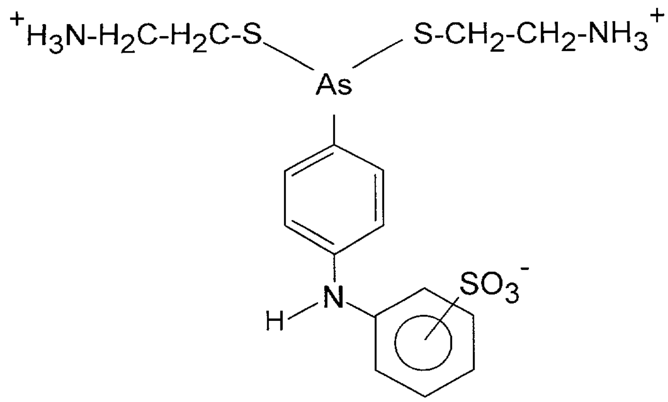
Derivative of B conjugated with H:

N) 

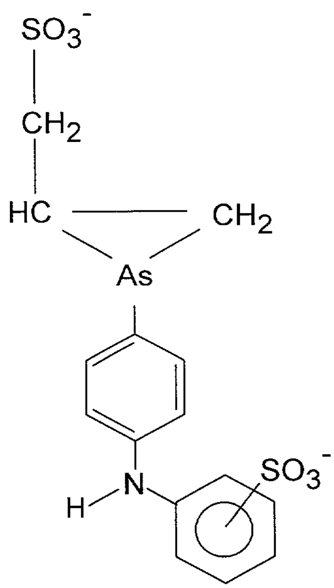
Derivative of C conjugated with F:

O) 

Derivative of C conjugated with G:

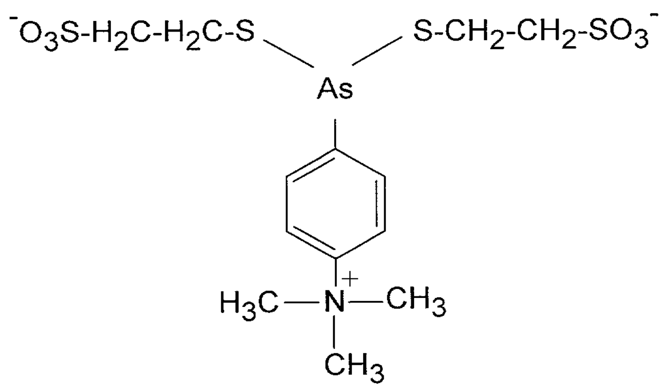
P) 

Derivative of C conjugated with H:

Q) 

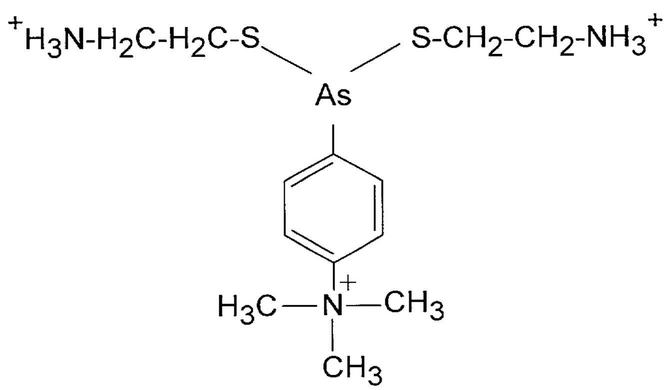
Derivative of D conjugated with F:

R)



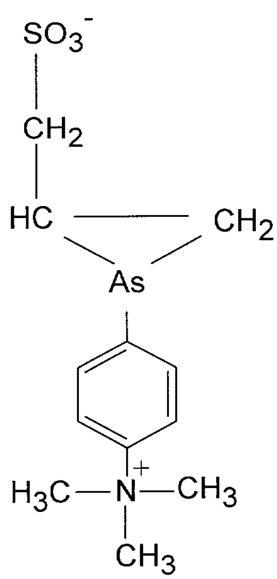
Derivative of D conjugated with G:

S)

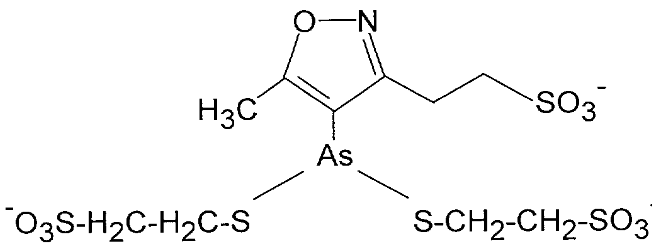


Derivative of D conjugated with H:

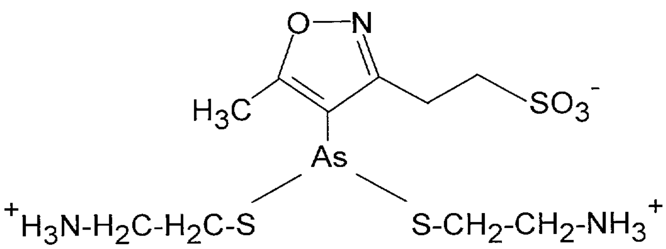
T)



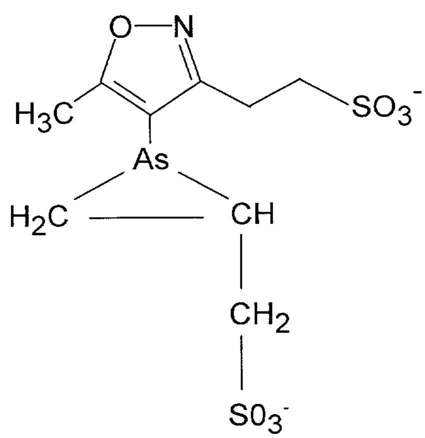
Derivative of E conjugated with F:

U) 

Derivative of E conjugated with G:

V) 

Derivative of E conjugated with H:

W) 

A novel class of membrane-impermeable arsenoxide derivatives is generated on this basic principle. Conjugation of a membrane-impermeable compound to the arsenical via (di)thiols makes the compound membrane-impermeable until the compound reacts with the vicinal dithiol of PDI and the original adduct is reduced and released extracellularly. By

conjugating ligands F-H to form membrane-impermeable PAO* derivatives, the derivative is fully restricted to the outside of the cells and non-toxic. The number of anti-trypanosomal drugs have been generated on this chemical principle (Eur. J. Biochem, 221:285-95, 1994), but with one important difference: these anti-trypanosomal drugs need to enter the cells, so both sides of the active site arsenical are made extra membrane-permeable.

EXAMPLE IV

Method for Whole Blood Measurement of Inhibitor-Activity

Inhibitor in whole blood can be measured as follows:

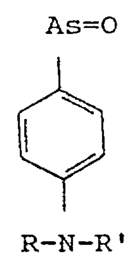
Peripheral venous blood was diluted 1:10 with HHB buffer and treated with 1 μM PAO for 20 min. at 37°C. L-selectin expression was measured by FACScan analysis (*supra*) of LDS-751 and Leu8-FITC labelled cells (FIG. 4). For HIV treatment, activity is preferably measured by L-selectin expression on lymphocytes, rather than neutrophils.

Induction of L-selectin shedding in whole blood is useful as an assay to determine the inhibitor concentration required to block, e.g., leucocyte or lymphocyte PDI. This whole-blood *ex-vivo* assay is preferable to assays of purified leucocytes or lymphocytes for clinical use of the inhibitors.

WHAT IS CLAIMED IS:

1. A membrane-impermeable inhibitor of protein disulfide isomerase (PDI).

2. An inhibitor according to Claim I of the formula



wherein at least one of R and R' is a charged ligand containing from 1 to 6 carbon atoms.

3. An inhibitor according to Claim 2, wherein the charged ligand contains at least one sulfonate group.

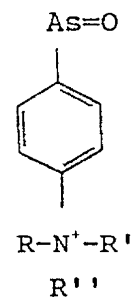
4. An inhibitor according to Claim 2, wherein the ligand is a straight chain or branched alkyl group containing 1, 3, 4, or 6 carbon atoms and at least one sulfonate group.

5. An inhibitor according to Claim 2, wherein the ligand isan aryl group containing at least one sulfonate group.

6. The inhibitor of Claim 5, wherein the sulfonate group is attached to a ring carbon atom.

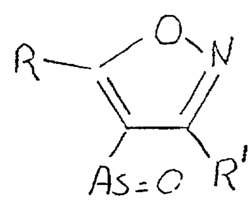
7. The inhibitor of Claim 6, wherein the sulfonate group is attached to the ring carbon atom via a C_1-C_6-alkylene group.

8. An inhibitor according to claim 1 of the formula



wherein R is H or alkyl.

9. An inhibitor according to Claim 1 of the formula



wherein at least one of R and R' is a charged ligand.

10. An inhibitor according to Claim 9, wherein the charged ligand contains at least one sulfonate group.

11. An inhibitor according to Claim 2 or 9, wherein one of R or R' is an uncharged H or C_1-C_6-alkyl ligand.

12. A method for inhibiting PDI by exposing cells expressing PDI to a compound according to any one of Claims 1-8 in an amount sufficient to inhibit PDI activity.

13. The method of Claim 12, wherein PDI activity is measured by assaying L-selectin shedding from leucocytes or lymphocytes.

14. A method for treating a mammal for a viral infectionpropagated by PDI-mediated virion entry into host cells comprising administering to the mammal phenylarsine oxide (PAO) or a compound according to any one of Claims 1-8 in an amount sufficient to inhibit viral propagation.

15. The method of Claim 14, wherein the viral infection is an HIV infection.

16. A method for measuring the potency of a potential PDI inhibitor comprising assaying cell L-selectin shedding according to the Snezna L-Selectin Assay as a direct measure of inhibition potency.

17. The method of Claim 16, wherein leucocytes or lymphocytes are exposed to a potential PDI inhibitor, tagged with a labeled anti-L-selectin antibody and assayed for released L-selectin.

18. The method of Claim 16, wherein leucocytes or lymphocytes are prelabeled with a detectable anti-L-selectin antibody, contacted with a potential PDI inhibitor, and assayed for released selectin.

19. A method for determining optimum blood concentrations of a PDI inhibitor for treatment of a mammal for a viral infection according to Claim 14 or 15, comprising admixing a blood sample with PDI inhibitor and assaying for leucocyte L-selectin shedding.

ABSTRACT OF THE DISCLOSURE

The invention provides anti-thiol reagents which inhibit enzyme activity of cell-associated protein disulfide isomerase (PDI) by oxidizing or blocking PDI active site vicinal thiol groups which normally participate in disulfide bond rearrangement of PDI substrates. Inhibition of this PDI function is particularly useful in blocking PDI-mediated entry of HIV or other virions into a host cell, as well as inhibiting lymphocyte traffic through the lymph nodes. The invention further provides an assay for the identification of such PDI inhibitors based on the discovery that inhibitors of the invention also induce shedding of the leucocyte L-selectin adhesion molecule.

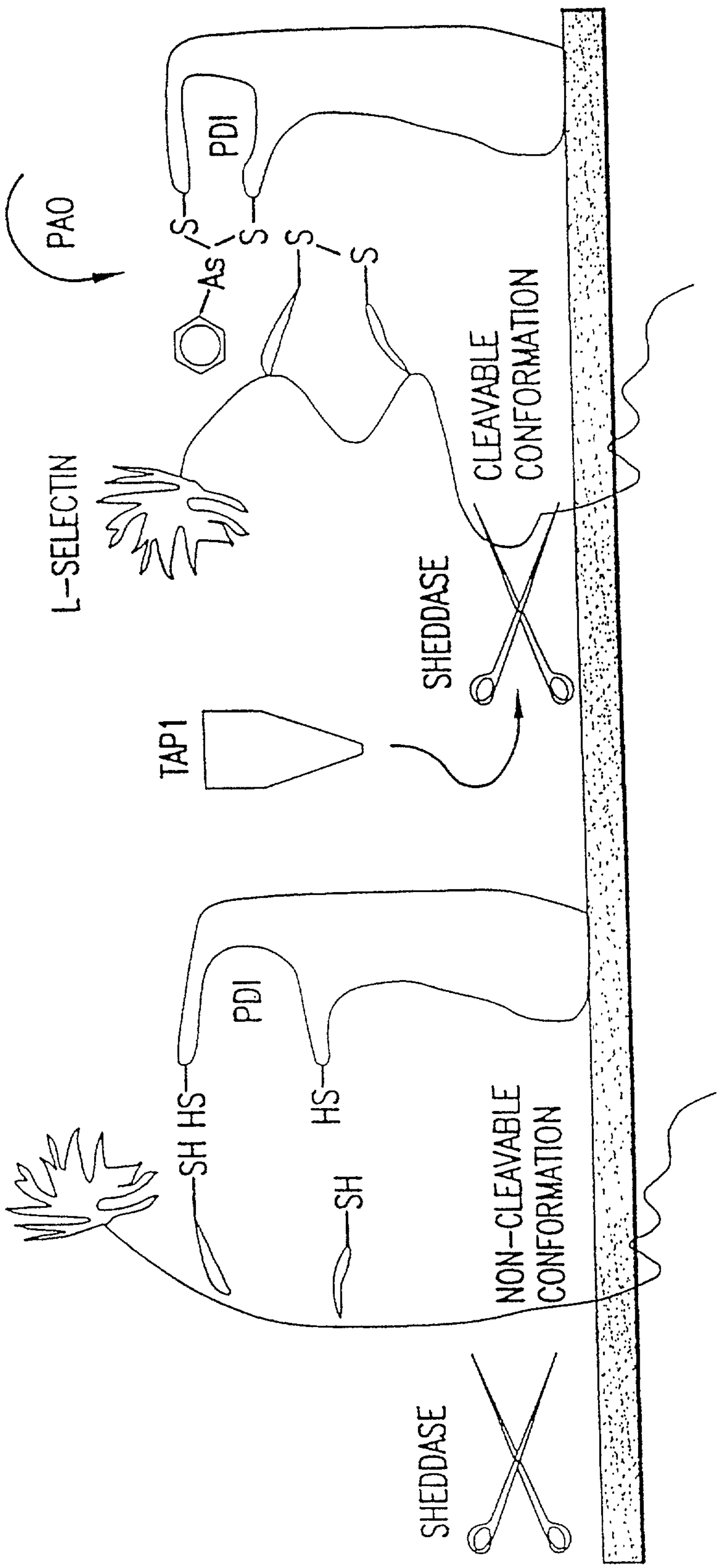
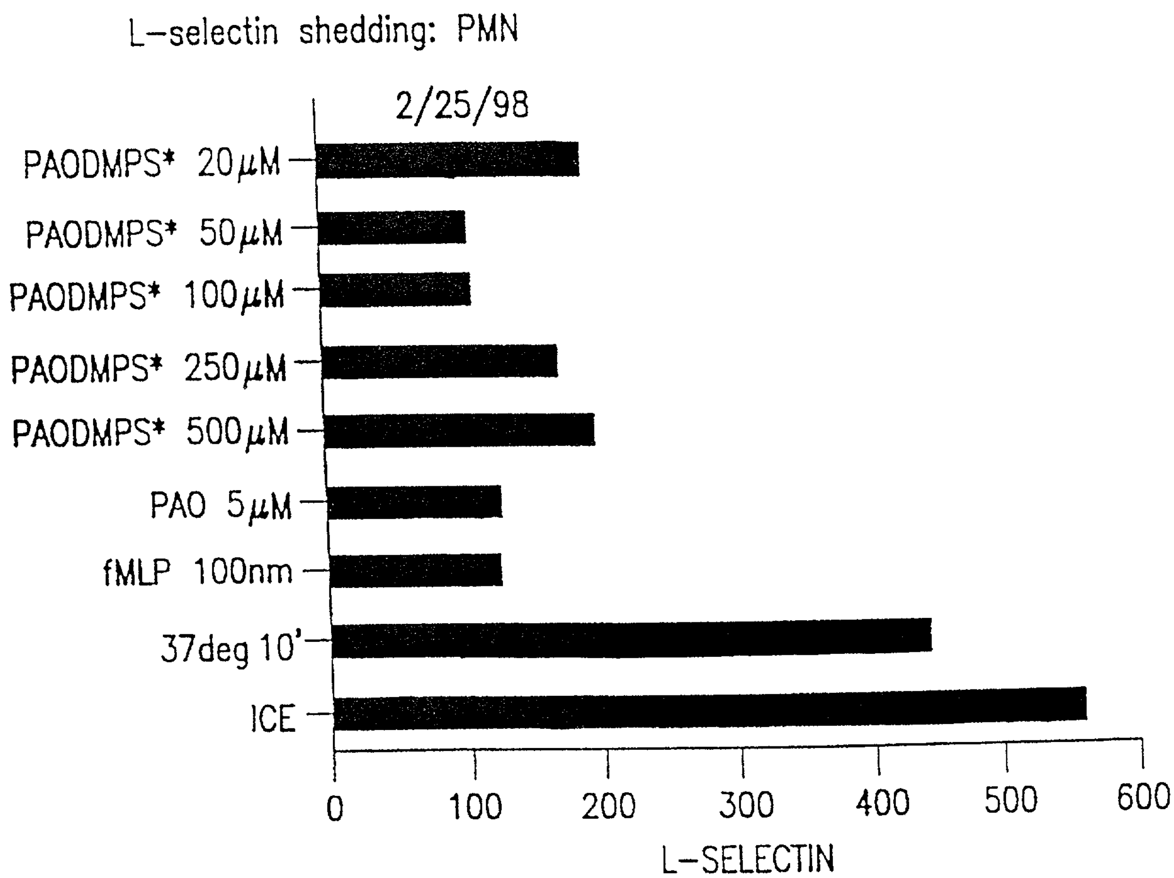

PAO
PDI
S
S
As
S
S
L-SELECTIN
CLEAVABLE CONFORMATION
SHEDDASE
TAP1
PDI
SH HS
HS
SH
NON-CLEAVABLE CONFORMATION
SHEDDASE

FIG.1



L-selectin shedding: PMN
2/25/98
PAODMPS* 20μM
PAODMPS* 50μM
PAODMPS* 100μM
PAODMPS* 250μM
PAODMPS* 500μM
PAO 5μM
fMLP 100nm
37deg 10'
ICE
0
100
200
300
400
500
600
L-SELECTIN

FIG.2

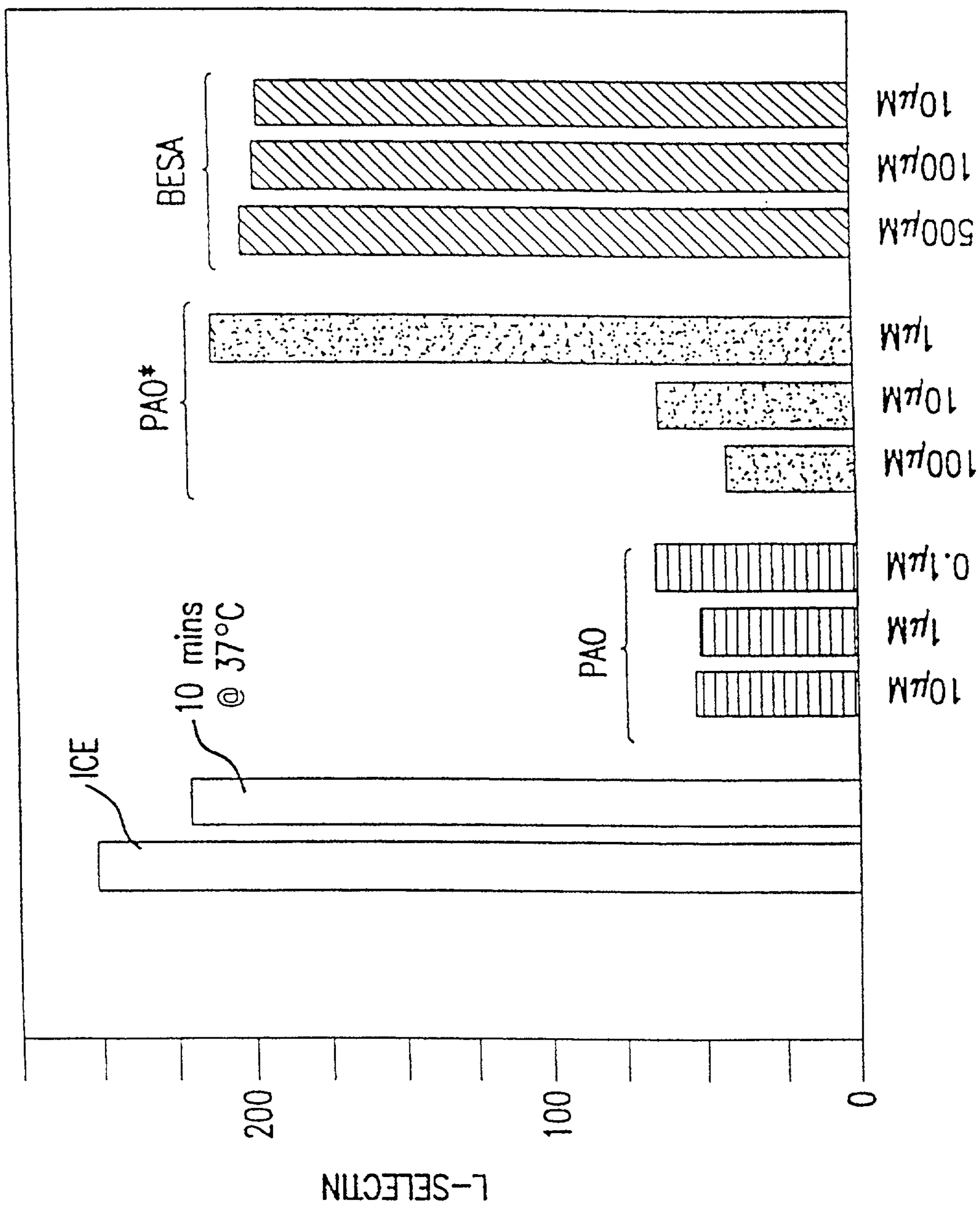

L-SELECTIN
0
100
200
ICE
10 mins @ 37°C
PAO
10μM
1μM
0.1μM
PAO*
100μM
10μM
1μM
BESA
500μM
100μM
10μM

FIG.3

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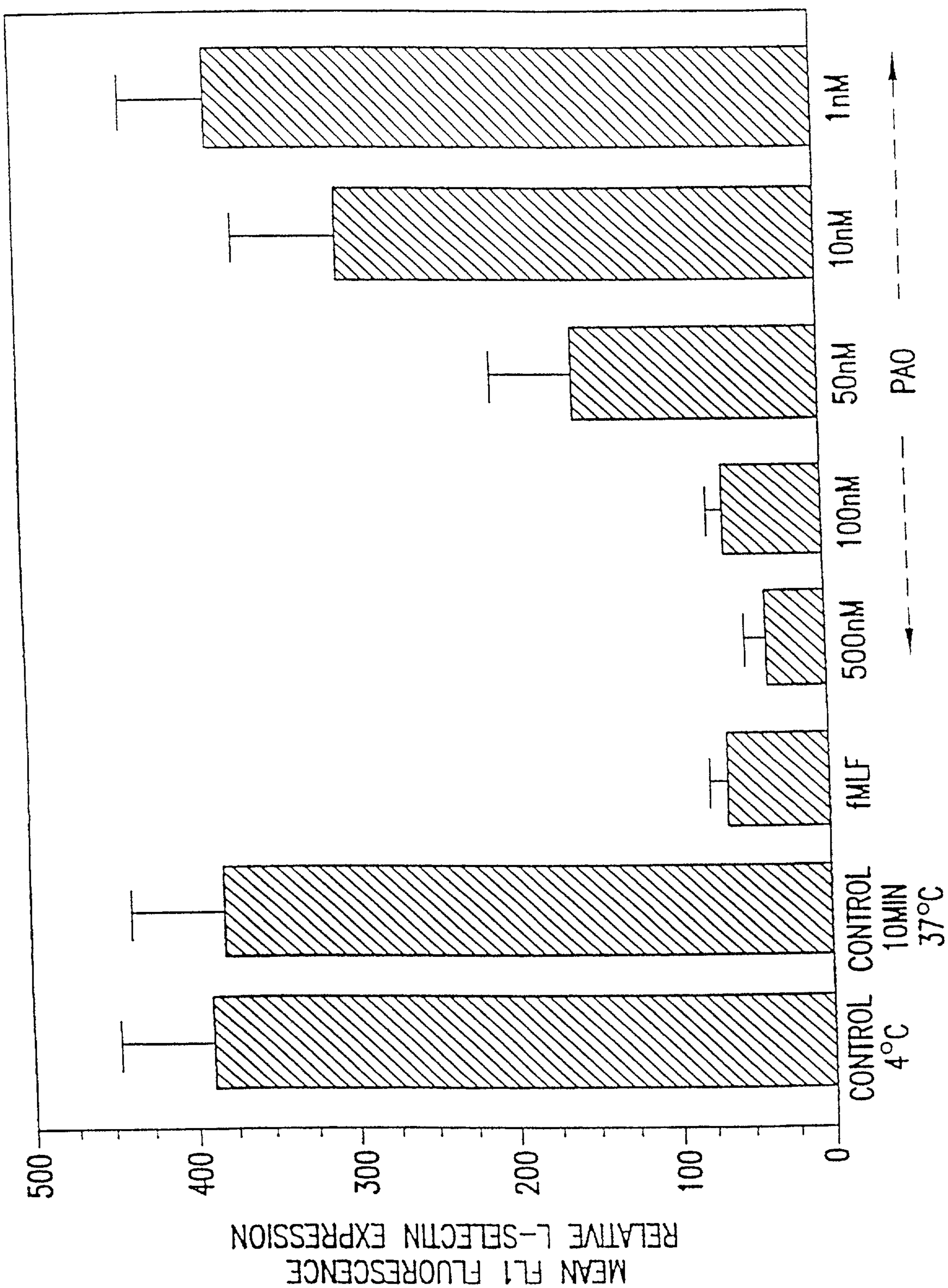

FIG.4A

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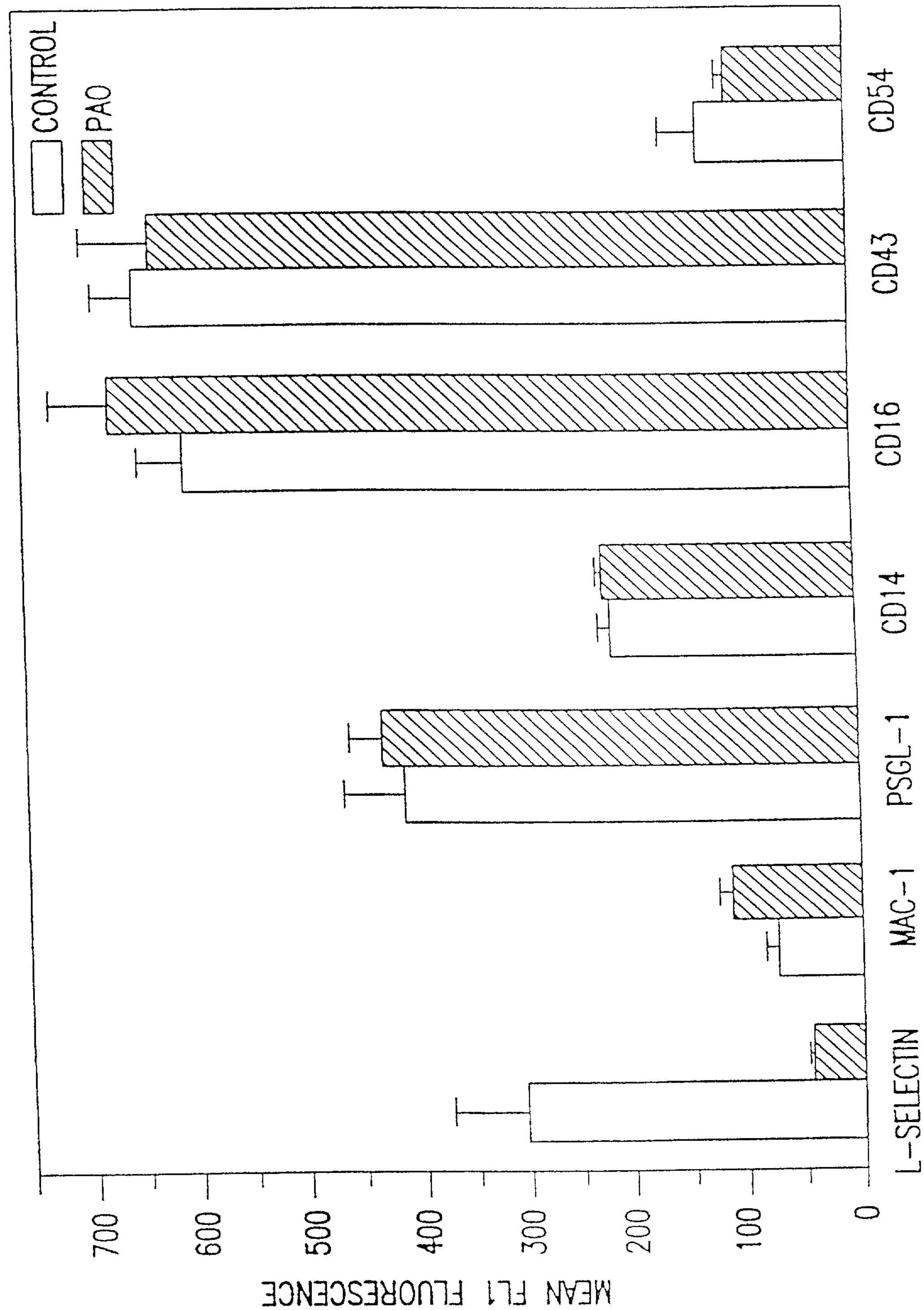

FIG.4B

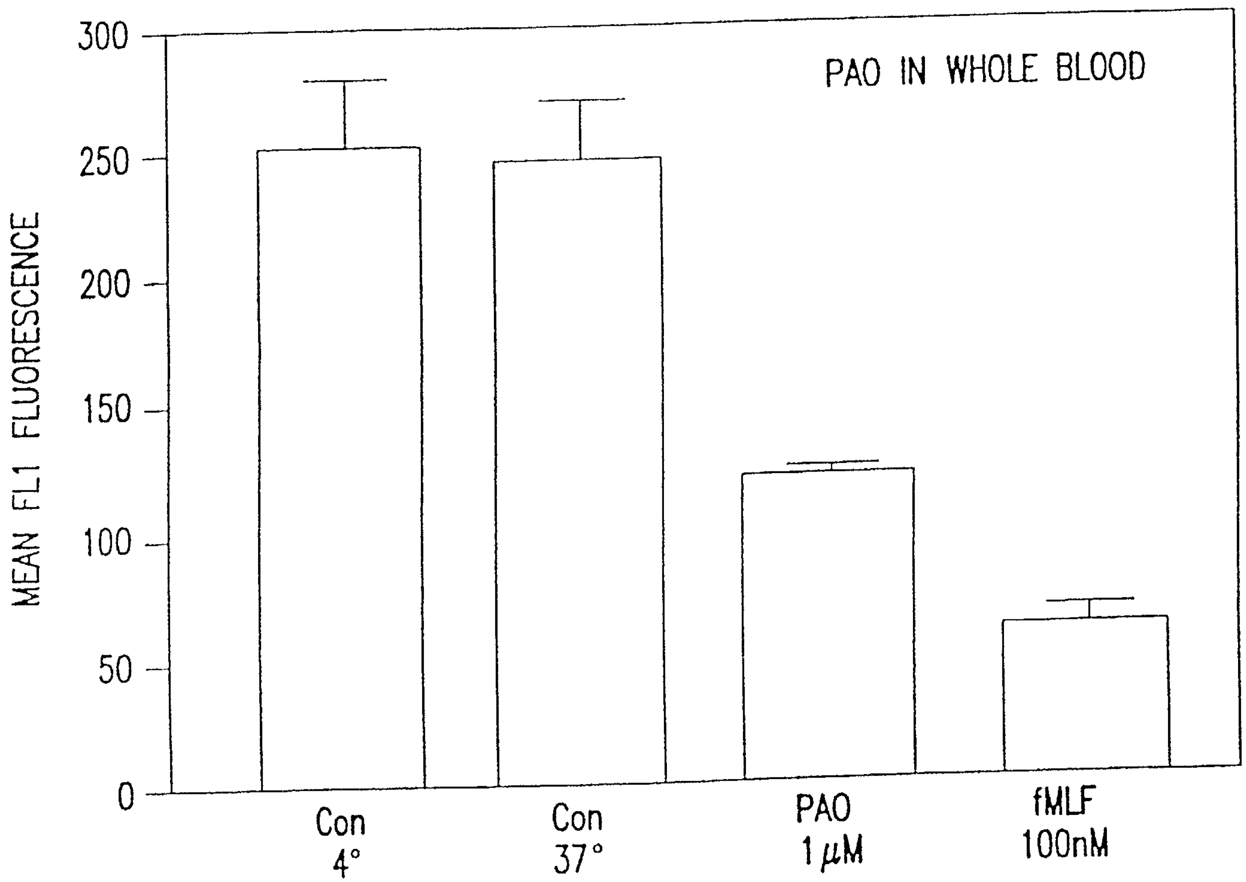
PAO IN WHOLE BLOOD
MEAN FL1 FLUORESCENCE
300
250
200
150
100
50
0
Con 4°
Con 37°
PAO 1 μM
fMLF 100nM

FIG.4C

Attorney Docket No. UNME-0054-1

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled: **INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE** the specification of which (check one):

- [X] is attached hereto.
- [ ] was filed on ______________ as Application Serial No. ______________, and was amended on ______________ (if applicable).
- [ ] was filed on ______________ as International Application (PCT) No. ______________ and was amended on ______________ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with *Title 37, Code of Federal Regulations, § 1.56(a)*. I hereby claim foreign priority benefits under *Title 35, United States Code § 119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which the priority is claimed.

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
			[ ] Yes [ ] No
			[ ] Yes [ ] No

I hereby claim the benefit under *Title 35, United States Code, § 120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, § 112*, I acknowledge the duty to disclose material information as defined in *Title 37, Code of Federal Regulations, § 1.56(a)* which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

APPLICATION NUMBER	FILING DATE	STATUS (Patented, Pending or Abandoned)
PCT/US98/09795	05-14-98	Published
60/046,487	05-14-97	Abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under *Section 1001 of Title 18 of the United States Code*, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I, We, hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Ajay A. Jagtiani, Registration Number 35,205.

Send correspondence to: JAGTIANI & ASSOCIATES
10379-B Democracy Lane
Fairfax, Virginia 22030

Telephone calls to: Ajay A. Jagtiani
(703) 591-2664

[X] *See following pages for additional joint inventors.*

1-00

Full Name of First or Sole Inventor ROGELJ, Snezna	*Citizenship* United States
Residence Address – Street 505 Mesa Loop	*Post Office Address Street* 505 Mesa Loop
City Albuquerque	*City* Albuquerque
State or Country New Mexico *Zip* 87801 NM	*State or Country* New Mexico *Zip* 87801
Date	*Signature* Snezna [signature]

2-a

Full Name of First or Sole Inventor SKLAR, Larry A.	Citizenship United States
Residence Address – Street 4000 Aspen Avenue, NE	Post Office Address Street 4000 Aspen Avenue, NE
City Albuquerque	City Albuquerque
State or Country Zip New Mexico 87112 N M	State or Country Zip New Mexico 87112
Date Nov 10, 1999	Signature

Full Name of First or Sole Inventor PALMER, Robert B.	Citizenship United States
Residence Address – Street 320 Sierra Place, NE	Post Office Address Street 320 Sierra Place, NE
City Albuquerque	City Albuquerque
State or Country Zip New Mexico 87108	State or Country Zip New Mexico 87108
Date Nov 10, 1999	Signature

Full Name of First or Sole Inventor	Citizenship
Residence Address – Street	Post Office Address Street
City	City
State or Country Zip	State or Country Zip
Date	Signature

09/437995

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
ROGELJ *et al.*	) Art Unit: To be assigned
Serial Number: 09/424,181	) (MISSING REQUIREMENTS)
Filed: May 14, 1998	) Examiner: John Anderson
For: INHIBITION OF CELL SURFACE PROTIEN DISULFIDE ISOMERASE	)

Assistant Commissioner of Patents
Washington, D.C. 20231

PETITION TO THE COMMISSIONER UNDER 37 CFR §§ 1.181 AND 1.48

Sir:

This is in response to the notification of missing requirements mailed on May 4, 2000, the period for response, which is set to expire on June 2, 2000. Applicant hereby petitions for the addition of inventor Robert B. Palmer to the application under 37 CFR § 1.48(a)(a).

STATEMENT

I, Snezna Rogelj, hereby declare that the error in inventorship occurred without any deceptive intention on my part.

Declared by: ______________________ Date 5/19/2000
Snezna Rogelj

STATEMENT

I, Robert B. Palmer, hereby declare that the error in inventorship occurred without any deceptive intention on my part.

Declared by ______________________ Date 5/31/2000
Robert B. Palmer

ATTY DOCKET NO. UNMX-0054-1

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled *INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE*

the specification of which (check one)

- [X] is attached hereto
- [ ] was filed on _______ as Application Serial No _______, and was amended on _______ (if applicable)
- [ ] was filed on _______ as International Application (PCT) No _______ and was amended on _______ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with *Title 37, Code of Federal Regulations § 1.56(a)*. I hereby claim foreign priority benefits under *Title 35, United States Code § 119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which the priority is claimed.

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under *Title 35, United States Code, § 120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, § 112*, I acknowledge the duty to disclose material information as defined in *Title 37, Code of Federal Regulations, § 1.56(a)* which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

APPLICATION NUMBER	FILING DATE	STATUS (Patented, Pending or Abandoned)
PCT/US98/09795	05-14-98	Published
60/046,487	05-14-97	Abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under *Section 1001 of Title 18 of the United States Code*, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Ajay A. Jagtiani, Registration Number 35,205

Send correspondence to: **JAGTIANI & ASSOCIATES**, 10379-B Democracy Lane, Fairfax, Virginia 22030

Telephone calls to: Ajay A. Jagtiani, (703) 591-2664

[X] See following pages for additional joint inventors.

Full Name of First or Sole Inventor ROGEL J. Snezna	*Citizenship* United States
Residence Address - Street 505 Mesa Loop	*Post Office Address Street* 505 Mesa Loop
City Albuquerque	*City* Albuquerque
State or Country New Mexico · *Zip* 87801	*State or Country* New Mexico · *Zip* 87801
Date 5/15/2000	*Signature*

Full Name of First or Sole Inventor	Citizenship
SKLAR, Larry A.	United States
Residence Address – Street: 4000 Aspen Avenue, NE	Post Office Address Street: 4000 Aspen Avenue, NE
City: Albuquerque NM	City: Albuquerque
State or Country: New Mexico Zip: 87112	State or Country: New Mexico Zip: 87112
Date: Nov 10, 1999	Signature: Larry A Sklar

Full Name of First or Sole Inventor	Citizenship
PALMER, Robert B.	United States
Residence Address – Street: 320 Sierra Place, NE	Post Office Address Street: 320 Sierra Place, NE
City: Albuquerque NM	City: Albuquerque
State or Country: New Mexico Zip: 87108	State or Country: New Mexico Zip: 87108
Date: Nov 10, 1999	Signature: [illegible]

Full Name of First or Sole Inventor	Citizenship
Residence Address – Street	Post Office Address Street
City	City
State or Country Zip	State or Country Zip
Date	Signature

09/438411

PTO/PCT Rec'd 10 NOV 1999

09/424181

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
	)
ROGELJ ***et al.***	)
	)
Serial Number: To be assigned	)**Art Unit:To be assigned**
	)
Filed: Concurrently herewith	)**Examiner: To be assigned**
	)
For: INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE	)
	)

ASSOCIATE POWER OF ATTORNEY

Sir:

I hereby appoint Mark Guttag, Registration Number 33,057, and Steven J. Prewitt, Registration Number 45,023 as my associate attorneys in the above-captioned application, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent and to transact all business in the Patent Office connected therewith.

However, please continue to address all future communications to the undersigned attorney at the following:

Ajay A. Jagtiani
JAGTIANI & ASSOCIATES
Democracy Square Business Center
10379-B Democracy Lane
Fairfax, Virginia 22030

Respectfully submitted,

Ajay A. Jagtiani
Registration Number 35,205

JAGTIANI & ASSOCIATES

Democracy Square Business Center
10379-B Democracy Lane
Fairfax, Virginia 22030
(703) 591-2664

November 9, 1999

Applicant or Patentee:	ROGELJ, *et al.*	Docket #:	UNME-0054-1
Serial or Patent Number:	To be assigned	Examiner:	
Filed or Issued:	Concurrently herewith	Art Unit:	
For:	INHIBITION OF CELL SURFACE PROTEIN DISULFIDE ISOMERASE		

VERIFIED STATEMENT (DECLARATION) BY A UNIVERSITY CLAIMING SMALL ENTITY STATUS UNDER 37 C.F.R. §§ 1.9(F) AND 1.27(C)

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
- ☒ an official of the University empowered to act on behalf of the concern identified below:

Name of Concern: **The University of New Mexico**
Address: **Hokona Hall, Room 357, Albuquerque, New Mexico, 87131**

I hereby declare that the above identified University qualifies as a small business concern as defined in *13 CFR 121.3-18*, and reproduced in *37 CFR 1 9(d)*, for purposes of paying reduced fees under *section 41(a) and (b) of Title 35, United States Code*, in that the number of employees of the concern, including those of its affiliates, **does not exceed 500 persons**. For purposes of this statement, **(1)** the number of employees is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and **(2)** concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the University identified above with regard to the matter described in:

- ☐ The specification filed herewith, with the title as listed above.
- ☒ The patent application identified above.
- ☐ The PCT International patent application identified above.
- ☐ The patent number identified above

If the rights held by the above identified University are not exclusive, each individual, concern or organization having rights to the invention must file separate verified statements averring to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under *37 CFR 1 9(c)* if that person made the invention, or by any concern who would not qualify as a small business concern under *37 CFR 1 9(d)*, or a nonprofit organization under *37 CFR 1 9(e)* Each person or organization having any rights in the invention is listed below:

- ☒ No such person, concern or organization.
- ☐ Each such person, concern or organization as listed below:

FULL NAME	☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization
ADDRESS	

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate *(37 CFR 1.28(b))*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under *section 1001 of Title 18 of the United States Code*, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which the verified statement is directed

NAME AND TITLE Annabelle D Quintana Associate University Counsel	DATE
ADDRESS University of New Mexico Patent Administration Office Hokona Hall, Room 357 Albuquerque, New Mexico 87131	SIGNATURE Annabelle Quintana

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

SCANNED, # 24

Application deficiencies found during scanning:

☐ Page(s)__________ of ____________________ were not present for scanning.
(Document title)

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(Document title)

☑ ***Scanned copy is best available.*** Declaration